Here we have developed a novel method to monitor for the first time with single molecule resolution the proton pumping activity of the quinol heme-copper oxidase, cytochrome bo_3 reconstituted in liposomes. using our recently developed arrays of surface tethered liposomes (*3-5*) and coupling electrochemistry with fluorescent microscopy allowed us for the first time to in situ activate and simultaneously monitor bo_3pumping activity in liposomes loaded with pH sensitive fluorescent reporters. Imaging in a massively parallel manner (10^3-10^4 liposomes) and at the single enzyme level allowed to directly observe, quantify the activity rates, abundance and lifetimes, of a plethora of interconverting long-lived (min) functional states. Parallel and multiplexed single vesicle and single molecule readout gave access to the full distribution of rates across the ensemble of proton pumps, and importantly how pH and membrane regulatory inputs modulate the average as well as the full fluctuation spectrum of bo_3 proton pump.

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Cyclic Nucleotide-gated Channels

1429-Pos Board B321

Distinct Contributions of CNGA3 and CNGB3 Subunits to Ligand-Specific Activation of Cone CNG Channels

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Cyclic nucleotide-gated (CNG) ion channels regulate the electrical activity of retinal photoreceptors, rods and cones, by sensing the light-induced changes of intracellular cGMP levels. Cone CNG channels consist of CNGA3 and modulatory CNGB3 subunits, both of which contain a cyclic nucleotide-binding domain (CNBD). CNGB3 subunits confer enhanced responses to cAMP and support several aspects of channel regulation. However, it is not fully understood how CNGB3 (and CNGA3) are specialized to contribute to ligand-specific activation of cone CNG channels. using patch-clamp recordings, we characterized several mutations located within the CNBD of CNGA3, each of which produced dramatic, ligand-specific effects on channel gating. In particular, D609M in CNGA3 reversed ligand selectivity, making cAMP a better agonist than cGMP, similar to equivalent mutations in paralogous channel subunits. These experiments suggest that mechanisms underlying ligand interaction with CNGA3 are well conserved. However, parallel mutations within the CNBD Cα-helix of CNGB3 had no effect on the ligand selectivity of heteromeric channels, consistent with the large decrement in sequence conservation in this region of CNGB3. CNGB3 appear to lack features supporting ligand discrimination. Next, we examined subunit contributions to ligand-dependent activation using CNBD "knock out" (R564E in CNGA3; R604E in CNGB3). CNGB3 R604E decreased relative cAMP efficacy, but only had a subtle effect on the cGMP activation for heteromeric channels (with wild-type or R564E CNGA3). In contrast, CNGA3 R564E caused an approximately 500-fold decrease in apparent cGMP affinity and nearly eliminated cAMP-dependent gating. Similar results were observed with analogous experiments using mutations of T565A in CNGA3 and T605A in CNGB3. Together, we propose that CNGA3 is the principal subunit mediating both ligand discrimination and ligand-dependent stabilization of the open state, while CNGB3 makes only a minor contribution to cGMP-dependent gating.

1430-Pos Board B322

Bacterial Roots and Branches of the HCN/CNG Family of Ion Channels: Phylogeny, Structure, and Implications for Eukaryotic HCN/CNG Structure and Function

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In gene, protein, and RNA families, it is typical for bacterial, archaeal, and eukaryotic members to form separate clusters. In this paper we report and analyze both phylogenetically and structurally a different pattern in the HCN/CNG family of ion channels, in which two subsets of bacterial members of the family clusters with eukaryotic members, rather than with the other bacterial members. The most parsimonious interpretation of the phylogeny is that this pattern is a consequence of horizontal gene transfer from a eukaryotic organism into a bacterium. We term the bacterial descendants of such transfer Eukaryotic-Like HCN/CNG's (ELHCN/CNG's). All of the ELHCN/CNG's have typical potassium channel selectivity filters. Thus in that sense they are more similar to HCN's than CNG's. However by more global similarity measures, they are roughly equally distant from the HCN's and CNG's, suggesting that the eukaryote-to-bacteria horizontal transfer was of a common ancestor to both the HCN's and CNG's. Our phylogenetic analysis further suggests that among the bacteria, subsequent spread of these two subsets was as much by horizontal transfer as by lineal descent. One possible mechanism for such transfer is amoeba, for which there is evidence that they engage in horizontal transfer with bacteria, and also facilitate horizontal transfer among bacteria. The ELHCN/CNG's may be useful biophysical and functional models for eukaryotic members of this family, especially because they share with the eukaryotic members a long C-linker between the inner helix of the permeation pathway and the ligand binding sites in the cyclic nucleotide binding domain. We present a model-built structure of one of the ELHCN/CNG's, which suggests a mechanism for coupling of ligand binding with channel opening.

1431-Pos Board B323

Inactivated spHCN Channel has a Decreased Binding Affinity for Camp

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In response to both voltage and ligand, HCN channels play important physiological roles in the brain and the heart. HCN channels are activated by membrane hyperpolarization and the direct binding of intracellular cAMP. The spHCN channel was cloned from Sea Urchin and belongs to the HCN channel family. Different from the mammalian HCN1-4 channels, the spHCN channel exhibits strong inactivation in the absence of cAMP. Application to cAMP to WT spHCN channel abolishes the inactivation. Interestingly, a previously identified point mutation near the inner activation gate in S6, F459L, makes the channel behave just like the mammalian HCN channels with any inactivation. Taking advantage of the patch-clamp fluorometry technique, we set out to investigate the dynamic, activity-dependent cAMP binding during spHCN channel gating. Surprisingly, during channel activation, we observed a decrease in cAMP binding, which is directly opposite to the observation with the HCN2 channel. Conversely, in the spHCN/F459L mutant channel, we observed an increase in cAMP binding during channel activation, which is similar to that observed in HCN2 channel. These observations provide new insights into the intriguing communication between the voltage-dependent and ligand-dependent gating in HCN channels.

1432-Pos Board B324

Counting of Ion Channels on a Membrane Patch Aided by Patch-Clamp Fluorometry

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Direct estimation of the number of channels on a membrane path is important for channel biophysics. It is a classical question and has been addressed elegantly by previous studies. Especially, pioneering researchers took the advantage of fluctuations in membrane conductance caused by ion channels opening and closing. The number of channels, the single-channel current, and the probability of the channel opening can be obtained using the method of non-stationary or stationary fluctuation analysis. Here, we developed a method to count the number of channels by simultaneous electrical recording of channel opening and optical recording of the fluorescence from the green fluorescent protein(GFP) tagged to the channel. Based on the number of channels and the macroscopic current, we first tuned this method using the cyclic-nucleotide gated (CNG) channel, of which the single channel conductance and open probability are well characterized. Then we applied the I-F relationship to the hyperpolarization-activated, cAMP-regulated HCN channel, of which the estimation of single channel conductance has been controversial. We estimated that the number of channels on a piece of membrane patch could read 10,000 to 20,000 and the single channel conductance for mHCN2 channel is about 1.82 pico Siemens.

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Properties of Single HCN2 Channels Expressed in Xenopus Oocytes

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Hyperpolarization activated cyclic nucleotide-modulated (HCN) channels mediate rhythmic electrical activity in specialized brain neurons and cardiomyocytes. The channels are non-specific cation channels that are activated by hyperpolarizing voltage. Activation is enhanced by the binding of cAMP to cyclic nucleotide binding domains in each of the four subunits. In mammalians four isoforms of HCN channels have been identified (HCN1-4). The single-channel conductance of HCN channels has been described first in native cardiac channels (DiFrancesco, Nature, 1986). Its value was determined to be only ~1 pS which is unusually small for a voltage gated cation channel. Surprisingly, in recombinant HCN2 channels a much larger conductance of ~35 pS was